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[COMMUNICATION]

The Confocal Laser Scanning Microscope as a Tool for Studying Xanthophores of the Swordtail (*Xiphophorus helleri*)

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ABSTRACT—The morphology of xanthophores on scales of the swordtail, *Xiphophorus helleri,* was studied using bright-field light, differential interference contrast (DIC) and confocal laser scanning microscopies (CLSM). Under light microscopy, xanthophores were indistinct with diffused cell outline. Improved imaging, showing gradient shaded relief-like images of xanthophores, was obtained under DIC. The CLSM produced the clearest images of xanthophores and also intracellular details as these cells were autofluorescent when scanned with the argon ion laser beam. The cell nuclei were non-autofluorescent and thus appeared dark. Selected portions of a cell can be highly magnified to show intracellular structures using the CLSM computer software. Also, serial optical tomographic sections of xanthophores with the CLSM enabled studies of intracellular structures present at different depths of the cells, thus permitting reconstruction of three-dimensional cell models. Therefore, use of the CLSM is highly recommened to complement bright-field light and DIC microscopies for morphological studies of xanthophores and other autofluorescent cells.

INTRODUCTION

Confocal laser scanning microscopy is an emrging technology with numerous applications in cell biology and cytochemistry. The confocal laser scanning microscope (CLSM), used primarily for visualization of fluorescent molecules (which can be either autofluorescent or fluorochromelabeled antibodies and ion sensitive dyes), permits more precise observations of sub-cellular structures, such as microtubules, centrosomes, chromosomes and nuclei, than could be achieved with conventional epifluorescent microscopy [1–3]. There is also improved spatial and lateral imaging of fluorescent specimens with overlapping structures such as eggs and embryos [3].

During confocal laser scanning, a specimen is illuminated with a small diffraction limited spot from a focused laser beam. Fluorescent signals from the illuminated spot, viewed with spatially restricted imaging optics, are descanned, spatially filtered and detected by a photomultiplier prior to image assembly in an integral image processor and display on a high-resolution video monitor [1]. A confocal image is then formed with improved rejection of out-of-focus information and greater resolution than conventional light microscopy [1–3].

The CLSM can contribute greatly to the field of cellular tomography. Thick fluorescent stained specimens, for example; sea urchin eggs, microtubules of HeLa cells, plasmacytoma cells, *Drosophila* salivary glands, nematodes and chick embryos can be optically sectioned to generate high resolution images of serial tomographic sections without the need for laborious microtome sectioning of fixed or frozen specimens [1, 4, 5]. Specimens are sectioned along the X-Y plane by the laser beam at varying preset thickness to produce Z-Series optical sections at different levels. A smoothly continuous three-dimensional model can be reconstructed from these serial

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sections [5].

Brightly colored vertebrates, especially fishes, possess various chromatophore types such as melanophores (black), erythrophores (red), xanthophores (yellow), xantho-erythrophores (yellow with red periphery), leucophores (white) and iridophores (reflecting) [6–9]. Chromatophore morphology is usually studied with conventional light microscopy and ultrastructural details with electron microscopy [9–11].

In this study, three different microscopy techniques; bright-field light, differential interference contrast (DIC) and confocal laser scanning were employed to examine the morphological characteristics of xanthophores of fancy color varieties of the swordtail, *Xiphophorus helleri*. Studies of xanthophores with light and DIC microscopes have always produced indistinct images due to the dense carotenoid pigment content [6, 8, 9]. Therefore, the main focus of this study was the application of the special functions of the CLSM. Since xanthophores possess fluorescent pteridines in pterinosomes [10–13], they are ideal specimens for CLSM investigations which have several distinct advantages over bright-field light and DIC techniques.

MATERIALS AND METHODS

Scales from the dorsal and ventral regions of the Golden and Neon varieties of swordtail were detached with fine forceps and individually mounted in teleost physiological saline, TPS (6.5 g NaCl, 0.4 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$ and 0.15 g $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water, pH 7.3) on glass slides. An Olympus BH2 binocular light microscope, at 200 to 1000× magnifications, was used for conventional bright-field light microscopy studies. The BH-2 microscope was also fitted with a Nomarski attachment (BH2-NIC) for DIC studies.

The BIO-RAD Lasersharp MRC-500 Confocal Laser Scanning Microscope (BIO-RAD Microscience Div., UK), consisting of a computer controlled laser scanner assembly attached to a Nikon Optiphot microscope, was used for in-depth studies of autofluorescent granules and intracellular structures in xanthophores present on the scales. The CLSM had an argon ion laser scanning in multiline mode but a special interference filter assembly, comprising 488 nm (blue) excitation and 515 nm (yellow) emission filters, was used to select specific lines. The CLSM laser beam, of 488 nm wavelength, was used at different magnifications for scanning the xanthophores.

Xanthophores were optically sectioned along the X-Y plane by the laser beam at various preset thickness (1–2 μm) to generate Z-Series optical tomographic sections at different depths of the cells. Photomicrographs of xanthophores and images collected by the CLSM were taken using the Olympus BH2 microscope with PM-10AD Exposure Unit, and Polaroid Freeze-frame Photomicrographic System, respectively. Kodak TMX-100 print film was used for the photographs.

RESULTS AND DISCUSSION

Xanthophores of swordtails were difficult to distinguish as discrete cells with distinct cell outline

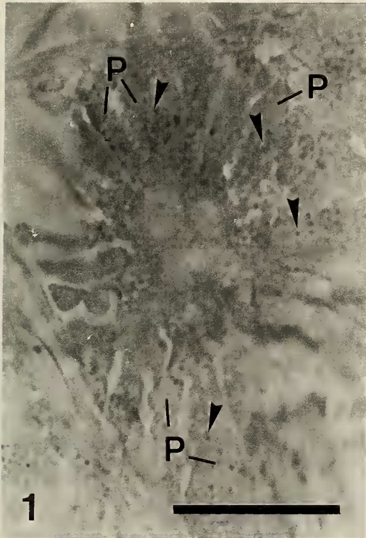
Fig. 1. High magnification light microscope photomicrograph of a xanthophore on a dorsal scale of a swordtail (Golden variety), showing indistinct cell margin and pterinosomes (arrowheads) in the cell processes (P). Scale bar, 10 μm.

Fig. 2. Low magnification differential interference contrast photomicrograph of a dorsal scale from a swordtail (Golden variety), depicting interlinking processes (arrowheads) between a xanthophore (X) and adjacent xantho-erythrophores (XE). Scale bar, 50 μm.

Fig. 3a. Low magnification confocal laser scanning image of three xanthophores on a dorsal scale of a swordtail (Neon variety), showing autofluorescent pterinosomes (arrows) and non-autofluorescent nucleus (N). Scale bar, 50 μm.

Fig. 3b. High magnification view of the outlined region in Fig. 3a, illustrating uniformly intense autofluorescent pterinosomes concentrated in high densities around the cell nuclei (N) and scattered ones in lower densities (arrows) in the dendritic cell processes. Scale bar, 25 μm.

Fig. 4. Confocal laser scanning images of Z-Series optical tomographic sections (a-h) at 1.5 μm depth intervals of a xanthophore on a ventral scale of a swordtail (Golden variety). Scale bar, 50 μm.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P
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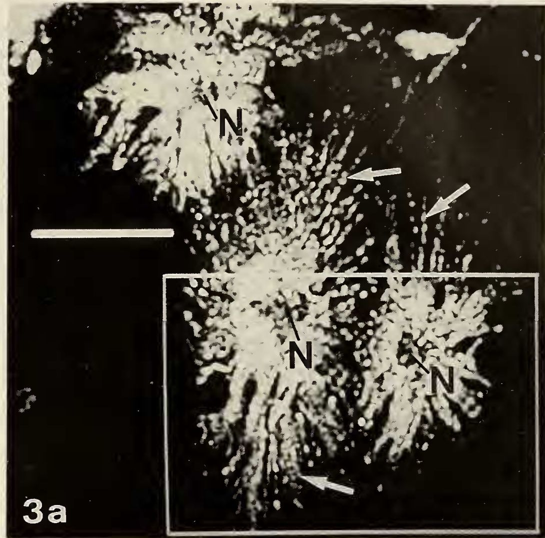
1


N
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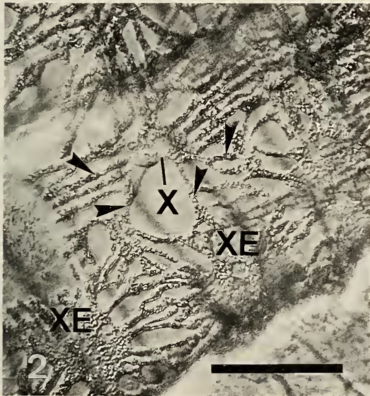
3a


X
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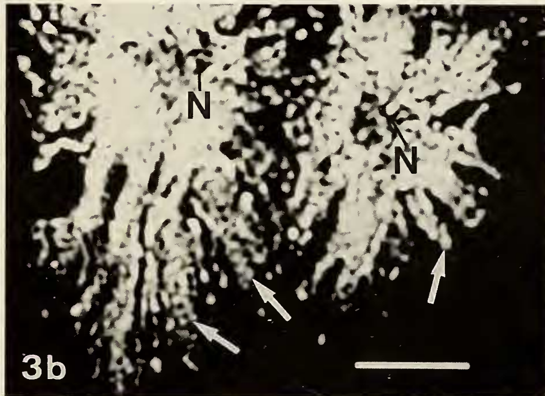
2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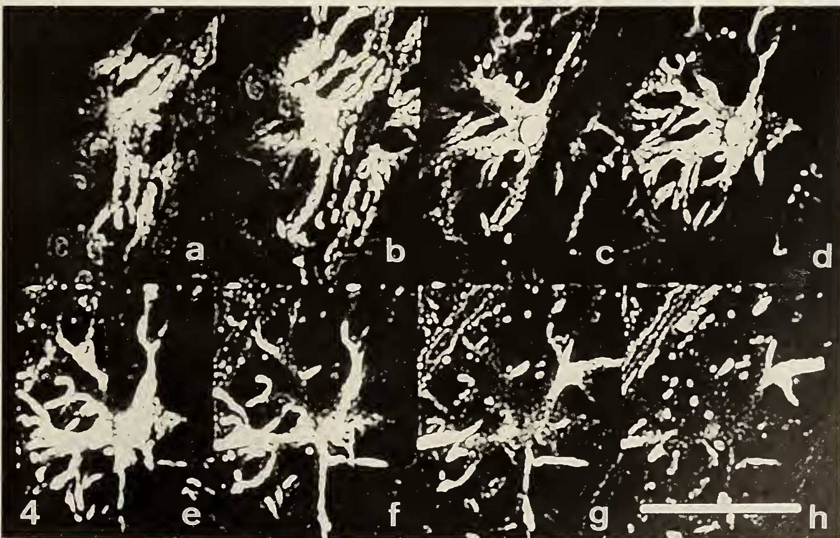
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under bright-field light microscopy due to a homogeneously distributed dense central yellow pigment content and diffused cell margin. At high magnifications (Fig. 1) and under DIC (Fig. 2), circular carotenoid vesicles and pterinosomes were observed to be scattered in the extended cell processes. These pterinosomes were observed by Matsumoto [10] to be organelles possessing a three-layered limiting membrane and inner lamellae which appeared whorl-like due to a concentric arrangement of parallel membranes. The dense yellow pigment in xanthophores was described by Goodrich *et al.* [8] and Matsumoto [10] as carotenoid pigments.

The CLSM proved immensely useful as xanthophores were autofluorescent (Fig. 3a) when scanned with the argon ion laser beam [14, 15]. The capacity for autofluorescence confirmed Matsumoto's [10], Matsumoto and Obika's [11], and Valenti's [12] findings that visible colored and colorless fluorescent pteridines were present in these cells. According to Matsumoto [10], xanthophores lack brightly-colored pteridines while their colorless pteridine content varies both in quality and quantity. Consequently, colorless pteridines such as xanthopterin, isoxanthopterin and biopterin [9, 10–12], and colored ones like yellow sepiapterin [13] might be the primary source of autofluorescence in xanthophores. Conversely, carotenoids such as lutein, zeaxanthin and β-carotene, studied by Goodrich *et al.* [8], Valenti [12] and Rempeters *et al.* [16] in *X. helleri* and *X. maculatus,* were not thought to contribute to autofluorescence in these cells.

The actual shapes of xanthophores can be elucidated from the distribution and arrangement of autofluorescent pterinosomes in the cells (Fig. 3a). High densities of pterinosomes caused intense autofluorescence at the cell center as opposed to the scattered distribution of autofluorescent pterinosomes in the cell processes [10, 11]. The CLSM micrograph showed xanthophores having numerous fine dendritic processes unlike the diffused cell outline produced by light microscopy (Fig. 1).

Intracellular organelles such as nuclei were non-autofluorescent, thus appearing dark and oval-shaped in contrast to the autofluorescent pterinosomes in the cytoplasm (Figs. 3a, b). Sections of xanthophores could be selected and highly magnified with the CLSM computer software for precise and detailed intracellular studies (Fig. 3b). Furthermore, dimensions such as length and width of cells or sub-cellular structures could be easily measured using this software.

Swordtail xanthophores were commonly observed to form interlinkages through the fusion of their long dendritic processes to adjacent xanthophores and xantho-erythrophores. These tip-to-tip interconnections between the two chromatophore types were best observed with the DIC attachment (Fig. 2) as the CLSM depicted overlapping and interconnecting autofluorescent processes as being similar. Interlinking xanthophores were not shown with light microscopy.

Z-Series optical tomographic sectioning of xanthophores on fresh scales with the CLSM was performed without the laborious tasks of processing and microtome sectioning of scales. Serial optical sectioning at a preset thickness (1–2 μm) permitted the observation of cells at consecutive intracellular levels. Fig. 4 depicted a xanthophore scanned at eight successive levels. From these images, xanthophores could be made out as flattened cells with long, fine dendritic processes projecting in all directions from the cell body. Hence, three-dimensional cell models could be reconstructed from the serial sections produced by the CLSM.

In conclusion, we recommend the use of the CLSM to complement conventional bright-field light and DIC microscopy techniques for fast and comprehensive morphological studies of fish xanthophores and other autofluorescent cells.

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